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Expression and Insulin-regulated Distribution of Caveolin in Skeletal Muscle

CAVEOLIN DOES NOT COLOCALIZE WITH GLUT4 IN INTRACELLULAR MEMBRANES*

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Caveolin is believed to play an important role in sorting processes, vesicular trafficking, transmembrane signaling, and molecular transport across membranes. In this study we have evaluated the expression and distribution of caveolin in skeletal muscle and its interaction with GLUT4 glucose carriers. Caveolin was expressed to substantial levels in muscle and its expression was regulated in muscle; aging and high fat diet enhanced caveolin expression in skeletal muscle and inversely, myogenesis down-regulated caveolin in L6E9 cells.

Under fasting conditions, most of caveolin was found in intracellular membranes and the caveolin present in the cell surface was found in both sarcolemma and T-tubules. Insulin administration led to a redistribution of caveolin from intracellular high density membrane fractions to intracellular lighter density fractions and to the cell surface; this pattern of insulin-induced redistribution was different to what was shown by GLUT4. These results suggests that caveolin is a component of an insulin-regulated machinery of vesicular transport in muscle.

Quantitative immunoisolation of GLUT4 vesicles obtained from different intracellular GLUT4 populations revealed the absence of caveolin which substantiates the lack of colocalization of intracellular GLUT4 and caveolin. This indicates that caveolin is not involved in intracellular GLUT4 trafficking in skeletal muscle.

Insulin stimulates glucose transport in skeletal muscle by a process that is characterized by an enhancement of V_{max} values (1–4). In parallel, insulin causes the recruitment of GLUT4, the main glucose carrier expressed in skeletal muscle, from an intracellular compartment to selective domains of sarcolemma and to T-tubules (5–11). Insulin is not the only effector that causes redistribution of GLUT4 in skeletal muscle; acute exercise has been demonstrated to cause recruitment of GLUT4 to the cell surface (12–14). There is not yet direct evidence on whether exercise recruits GLUT4 to similar cell surface domains and by translocation of similar intracellular GLUT4 compartments in skeletal muscle (12–14).

Important for the understanding of GLUT4 translocation is the knowledge of the proteins that colocalize with GLUT4 in the same compartment. A lack of colocalization of GLUT4 and TGN38 protein both in 3T3 adipocytes (15) and in rat skeletal muscle (11) has been substantiated, which indicates that the trans Golgi network is not a major site of the intracellular GLUT4 pool in adipocytes and in the muscle fiber. Recent studies have identified some of the proteins that colocalize in GLUT4-containing vesicles from rat adipocytes and skeletal muscle. Among the proteins that colocalize with GLUT4 in intracellular vesicles, the presence of phosphatidylinositol 4-kinase (16), VAMPs (17), SCAMPs/GTV3 (18, 19), gp160 (20–22), and some low molecular GTP-binding proteins including rab4 (23, 24) have been reported.

Caveolin is a principal component of the coat component of caveolae (25) and a major phosphoprotein in v-Src-transformed cells (26). Caveolin is believed to play an important role in sorting processes, transmembrane signaling and molecular transport across membranes (27). The relevance of caveolin to GLUT4 traffic has been recently investigated in 3T3-L1 and rat adipocytes (28, 29). Scherer *et al.* (28) have suggested that caveolae may play an important role in the vesicular transport of GLUT4 in 3T3-L1 adipocytes. In contrast, Kandror *et al.* (29) have concluded that caveolin has no direct structural relation to the organization of the intracellular glucose transporting machinery in isolated rat adipocytes. Here, we have studied the expression and distribution of caveolin in skeletal muscle, the regulation by insulin of caveolin distribution, and the possible role of caveolin on GLUT4 traffic.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-Labeled protein A and ^{125}I-labeled sheep anti-mouse antibody were purchased from ICN. ^{125}I-labeled goat anti-mouse antibody and enhanced chemiluminescence system (ECL) were from Amersham Corp. Immobilon poly(vinylidene) fluoride was obtained from Millipore. All electrophoresis reagents and molecular weight markers were obtained from Bio-Rad. γ-Globulin, wheat germ agglutinin, goat-anti mouse IgG and goat anti-mouse IgM coupled to agarose, and most commonly used chemicals were from Sigma.

Antibodies—Both monoclonal (1F8) and polyclonal (OSCRX) antibodies specific for GLUT4 were used in these studies. Monoclonal antibody 1F8 (30) was used for immunoisolation assays. Anti-GLUT4 (OSCRX) from rabbit was produced after immunization with a peptide corresponding to the final 15 amino acids of the carboxyl terminus (31). A rabbit polyclonal antibody against rat β_1-integrin was kindly given by Dr. Carles Enrich (University of Barcelona) (32). A rabbit polyclonal antibody against the α_2 component of Ca^{2+} channels (dihydropyridine receptors) (33) was obtained from Dr. Michel Lazdunski (Centre de Biochimie, Centre National de la Recherche Scientifique, Sophia Antipolis). Monoclonal antibody A-52 against Ca^{2+}-ATPase (34) was kindly

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donated by Dr. David H. MacLennan (University of Toronto). Monoclonal antibody against caveolin was obtained from Transduction Laboratories. Monoclonal antibody SY38 against synaptophysin was obtained from Boehringer Mannheim.

Animals and Tissue Sampling—For experiments dealing with the regulation of caveolin expression in skeletal muscle, 3- and 12-month-old male Wistar rats subjected to either regular chow diet (23% proteins, 5% fat, 50% carbohydrates, 4% cellulose, 6% minerals, w/w) or high fat diet for the last 6 months (13% proteins, 40% fat, 12% carbohydrates, 2% cellulose, 5% minerals, w/w) were used. For studies on the subcellular distribution of caveolin, male Wistar rats weighing between 250 and 300 g fed with Purina Laboratory Chow *ad libitum* were used. Animals were housed in animal quarters and maintained at 22 °C with a 12-h light, 12-h dark cycle. After an overnight fast, rats were anesthetized with sodium pentobarbital, and some were injected with insulin (intravenously, 10 units/kg of body weight) and D-glucose (intraperitoneally, 1 g/kg of body weight) 30 min prior to tissue removal. At that time, white portions of gastrocnemius and quadriceps muscles were rapidly excised and immediately processed.

Cell Culture—Rat skeletal muscle cell line L6E9 was grown in monolayer culture in α-minimal essential medium containing 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic (10,000 units/ml penicillin G and 10 mg/ml streptomycin). To obtain myotubes from both L6E9 myoblasts, subconfluents monolayers were maintained in 2% fetal bovine serum. After 4 days under these conditions they spontaneously differentiated into multinucleated myotubes.

Preparation of Total Membrane Fractions from Muscle—Total membrane fractions were obtained as described previously (35). In short, muscle was homogenized with a Polytron (setting 6, 2 × 30 s) in 10 volumes of ice-cold buffer containing 25 mM Hepes, 250 mM sucrose, 4 mM EDTA, 1 trypsin inhibitory unit/ml of aprotinin, 25 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin, pH 7.4. The homogenates were centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatants were adjusted to 0.8 M KCl, incubated at 4 °C for 30 min, and then centrifuged for 90 min at 200,000 × *g* at 4 °C to obtain the total membrane fractions. The membrane pellets were resuspended in homogenization buffer and repeatedly passed through a 25-gauge needle before storage at −20 °C. Total L6E9 membranes were prepared as follows. The cells were gently scraped in homogenization buffer (250 mM sucrose, 2 mM EGTA, 5 mM NaN_3, 20 mM Hepes, pH 7.4, containing 1 μM leupeptin, 1 μM pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) followed by 20 strokes with a Dounce type A homogenizer. The cell homogenate was centrifuged at 760 × *g* for 5 min to remove nuclei, mitochondria, and unlysed cells, followed by centrifugation at 190,000 × *g* for 60 min to obtain the total cell membranes. The pellets were resuspended in homogenization buffer. Proteins were measured by the method of Bradford (36) using γ-globulin as a standard.

Subcellular Fractionation of Rat Skeletal Muscle Membranes—The different cell surface and intracellular membrane fractions were isolated as reported elsewhere (37). Approximately 12 g of rat skeletal muscle were excised, weighed, minced, and initially homogenized with a Polytron at a low speed (setting 4, 2 × 5 s) in buffer A (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin, 1 μM leupeptin, pH 7.4; 1 g/4 ml). The homogenate was centrifuged for 20 min at 12,000 × *g* (9,000 rpm in SA-600 Sorvall rotor). The supernatant was collected and kept on ice. The pellet was resuspended in buffer A and centrifuged again for 20 min at 12,000 × *g*. The two supernatants were pooled and are referred to as the F1 fraction. The pellet was resuspended in buffer A and subjected to a high speed homogenization (Polytron at setting 6, 2 × 30 s). The homogenate was centrifuged for 20 min at 12,000 × *g*, and the supernatant was collected and referred to as F2. F1 and F2 fractions were incubated with 0.6 M KCl for 1 h at 4 °C and then pelleted for 1 h at 150,000 × *g* in a T-647.5 Sorvall rotor. The pellets from KCl-washed F1 and F2 fractions were then subjected to calcium loading in order to increase the density of sarcoplasmic reticulum vesicles (38). To this end, pellets were resuspended in buffer B (50 mM potassium phosphate, 5 mM $MgCl_2$, 150 mM KCl, pH 7.5) at a protein concentration of 2 mg/ml. Calcium loading was initiated by addition of 0.3 mM $CaCl_2$ and 2 mM ATP. After incubation for 20 min at room temperature, F1 and F2 fractions were kept on ice and centrifuged for 60 min at 150,000 × *g*. F1 and F2 pellets were resuspended in buffer C (20 mM Tris-HCl, 50 mM sodium pyrophosphate, 0.3 M KCl, 0.25 M sucrose, pH 7.2) and layered on top of a discontinuous density gradient consisting of 3 ml of 35%, 2 ml 29%, 2 ml 26%, and 2 ml of 23% (w/v) sucrose. After centrifugation for 12 h at 77,000 × *g* (25,000 rpm in a TH-641 Sorvall rotor), four protein fractions were separated from F1 and F2 fractions: fraction 23 on top of the 23% layer; fraction 26 from the interphase 23–26%; fraction 29 from the interphase 26–29%; fraction 35 from the interphase 29–35%. In some experiments, the pellet resulting from this centrifugation was also collected (pellet F1 and pellet F2). All the fractions were collected, diluted with 20 mM Tris-HCl, pH 7.4 and centrifuged for 60 min at 150,000 × *g*. Pellets were resuspended in 30 mM Hepes, 0.25 M sucrose, pH 7.4. Proteins were determined by the method of Bradford (36) using γ-globulin as a standard.

Isolation of Purified Sarcolemma and T-tubules by Wheat Germ Agglutination—To purify the surface membrane preparations, vesicles from the 23F2 fraction were treated with wheat germ agglutinin (39, 40). Surface membranes were resuspended at a protein concentration of 1 mg/ml in buffer D (50 mM sodium phosphate, 160 mM NaCl, pH 7.4) and mixed with an equal volume of 1 mg/ml wheat germ lectin (Sigma) in buffer D. Total volume of this mixture was 600 μl, and after 10 min incubation in ice, the solution was pelleted in a microcentrifuge for 1.5 min at 15,000 × *g* (13,000 rpm). The lectin agglutinated vesicles (W^+ fractions) were resuspended in buffer E (20 mM Tris-HCl, 0.250 M sucrose, pH 7.4) and centrifuged as described above. This procedure was repeated, and the resuspended pellets were then deagglutinated by incubation for 20 min at 0 °C in 500 μl of 0.3 M *N*-acetyl-D-glucosamine in buffer E. The deagglutinated suspension was centrifuged in a Microfuge for 1.5 min at 15,000 × *g* and the supernatant was pelleted at 150,000 × *g* for 60 min (in a TLS-55 Beckman rotor). The pellet was resuspended in buffer E and frozen in liquid nitrogen. The nonagglutinated vesicles (W^- fractions) were centrifuged for 60 min at 150,000 × *g*. The pellet was resuspended in buffer E and stored frozen.

Protocols of Vesicle Immunoisolation—Protein A-purified 1F8 antibody was coupled to acrylamide beads (Reacti-gel GF 2000; Pierce) at a concentration of 1 mg of antibody/ml of resin according to the manufacturer's instructions. Before usage, the beads were saturated with 1% bovine serum albumin in PBS[1] for at least 30 min and washed with PBS. Intracellular membranes were incubated with beads overnight at 4 °C. The beads were washed five times with PBS, and the adsorbed material was eluted with electrophoresis sample buffer, incubated for 5 min at 95 °C, cooled, and microcentrifuged. The supernatant fraction from the vesicle immunoadsorption assay and the immunoadsorbed extract were subjected to SDS-polyacrylamide gel electrophoresis.

In some assays, antibodies 1F8 (5–7 μg) and 3F8 (3 μl) were incubated overnight at 4 °C with goat anti-mouse IgG- or goat anti-mouse IgM-coupled to agarose (75 μl of bead volume). Beads were collected by a 6-s spin in a Microfuge and washed in PBS. Intact membrane preparations (15–25 μg of protein) were incubated with 1F8- or 3F8-Ig-agarose overnight at 4 °C in the absence of detergents (0.1% bovine serum albumin, 1 mM EDTA in PBS; final volume, 200 μl). The agarose beads and vesicles bound to them were collected by a 6-s spin in a Microfuge. The vesicles that were bound to the immobilized antibody were washed in PBS. The adsorbed material was eluted with electrophoresis sample buffer.

Isolation of Caveolin-rich Membrane Domains—Caveolin-rich membrane domains were prepared from rat skeletal muscle by a minor modification of an established protocol used for mouse lung (41). Rat skeletal muscle (400 mg wet weight) was minced with a scissors and homogenized with 2 ml of 0.1 M Tris buffer, pH 6.5, containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. Homogenization was carried out with a Polytron (three 5-s bursts). The homogenate was adjusted to 40% sucrose (w/w) by addition of 0.8 g of sucrose and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient (in 0.1 M Tris buffer, pH 6.5, lacking Triton X-100) was formed above the homogenate and centrifuged at 39,000 rpm for 12–16 h. Gradients were separated into 12 fractions.

Electrophoresis and Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis was performed on membrane protein following Laemmli (42). For GLUT1, GLUT4, TGN38, dihydropyridine receptors, Ca^{2+}-ATPase, β_1-integrin, tt28, nonreducing 8.5, 10, or 12% polyacrylamide gels were used. For immunodetection of β-subunit of the insulin receptors and dystrophin, samples were reduced with 100 mM dithiothreitol and run in 6% gels. Proteins were transferred to Immobilon as previously reported (43) in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% nonfat dry milk, 0.02% sodium azide in PBS for 1 h at 37 °C and were incubated with antibodies in 1% nonfat dry milk, 0.02% sodium azide in PBS. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished using ^{125}I-protein A for 4 h at room temperature. Detection of the immune com-

[1] The abbreviation used is: PBS, phosphate-buffered saline.

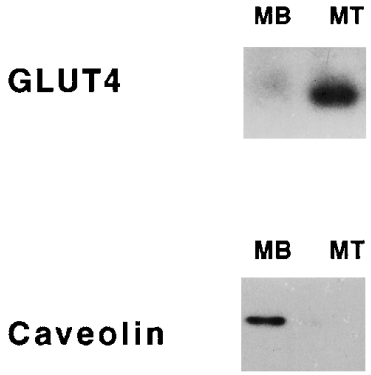

FIG. 1. **Expression of caveolin and GLUT4 in myoblast and myotube L6E9 cells.** Total membranes were obtained from myoblast and myotube L6E9 cells. The abundance of caveolin and GLUT4 was determined by immunoblot analysis by using specific antibodies (see "Experimental Procedures"). Representative autoradiograms from two to seven experiments are shown.

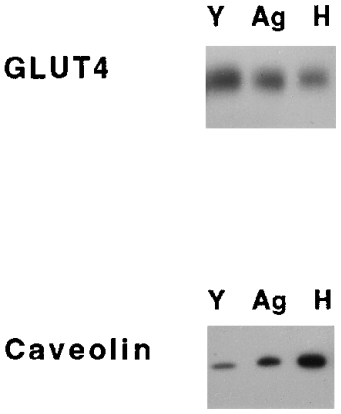

FIG. 2. **Effect of aging and high fat diet on caveolin and GLUT4 expression in skeletal muscle.** Total membrane proteins were purified from skeletal muscle obtained from 3-month-old rats (*Y*) and 12-month-old rats either subjected to regular diet (*Ag*) or to a high fat diet (*H*). The abundance of caveolin and GLUT4 was determined by immunoblot analysis by using specific antibodies (see "Experimental Procedures"). Representative autoradiograms from seven to 10 separate experiments are shown.

plex with monoclonal antibodies was performed using sheep anti-mouse ^{125}I-antibody. Antibody 3F8 was detected using horse radish peroxidase linked to goat anti-Ig M mouse secondary antibody and visualized using an ECL. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RESULTS

Expression of Caveolin in Rat Skeletal Muscle and Muscle Cells—In preliminary experiments, we found an abundant expression of caveolin in membrane preparations of rat skeletal muscle by Western blot analysis. It has been reported that adipose tissue contains a larger amount of caveolin protein than any other tissue (28). In keeping with this, we also found a very high level of expression of caveolin protein in membrane preparations from rat white adipose tissue; levels of caveolin present in rat skeletal muscle accounted for 10% of levels found in adipose tissue, when data were expressed per g of tissue (data not shown). The expression of caveolin has been previously reported in muscle cells (44–46). Based on this, we tried to determine whether caveolin expression was subjected to regulation in muscle. Initially, we studied whether myogenesis, a process that is associated with induction and repression of many different genes, leads to changes in cellular caveolin levels. To this end, we used cultured L6E9 cells under a myoblast or myotube phenotype. Muscle cell fusion and differentiation is triggered in L6E9 cells after depleting the culture medium of bovine fetal serum (47). Differentiation of muscle cells caused the induction of GLUT4 expression (Fig. 1), which is in keeping with previous observations in L6 cells (48, 49). Under these conditions, we found a substantial drop in the cellular levels of caveolin (Fig. 1).

In another set of experiments, we analyzed the muscle content of caveolin and GLUT4 in two different experimental conditions known to alter GLUT4 expression in skeletal muscle, *i.e.* aging and high fat feeding (50–52). To this end, we compared the expression of caveolin and GLUT4 in skeletal muscle from 3- and 12-month-old rats. In addition, we also studied the expression of proteins of 12-month-old rats subjected to a 6-month period of high fat feeding. The level of GLUT4 decreased 39% in muscle from aged rats compared to values found in the control group (Fig. 2). Furthermore, levels of GLUT4 further decreased (46% decrease) in muscle from aged rats as a result of high fat feeding (Fig. 2). In contrast, caveolin levels in skeletal muscle increased by 74% in aged rats compared to young rats (Fig. 2) and a high fat diet further increased the levels (99% increase compared to aged rats subjected to regular diet) (Fig. 2).

Distribution of Caveolin and GLUT4 in Skeletal Muscle—The distribution of caveolin and GLUT4 in the muscle fiber from overnight-fasted rats was next studied by using a previously described protocol of subcellular fractionation of skeletal muscle (37). β_1-Integrin, a sarcolemmal marker, and dihydropyridine receptor, a T-tubule marker, were mainly detected in cell surface membrane fractions (Fig. 3A). The cell surface membranes did not show contamination with sarcoplasmic reticulum vesicles as judged by the absence of sarcoplasmic Ca^{2+}-ATPase (Fig. 3*A*). In contrast, immunodetectable caveolin, showing an apparent molecular mass of 22 kDa, and GLUT4 were mainly localized in intracellular membrane fractions (Fig. 3*A*).

We have recently reported that agglutination of cell surface membrane fractions from skeletal muscle leads to the isolation of three distinct cell surface domains: sarcolemmal fraction 1 (SM1), sarcolemmal fraction 2 (SM2) a T-tubule fraction (37). In order to determine the localization of caveolin, comparatively to GLUT4, in the different cell surface membranes obtained from skeletal muscle, Western blotting of these proteins was performed on fractions SM1, SM2 and T-tubules. In keeping with previous observations (37), fractions SM1 and SM2 showed a high abundance of β_1-integrin, whereas the T-tubule fraction showed a low abundance of β_1-integrin (Fig. 3*B*). In keeping with previous observations, GLUT4 was present in all cell surface fractions, *i.e.* SM1, SM2, and T-tubules, and GLUT4 abundance was significantly greater in the T-tubule than in fractions SM1 or SM2 (Fig. 3*B*). Similar to the pattern of GLUT4 distribution, caveolin was found in substantial levels in all cell surface membranes analyzed (Fig. 3*B*). Caveolin and GLUT4 also showed a similar pattern of distribution in the different intracellular membranes obtained (Fig. 3*C*). Synaptophysin, a protein found in peripheral nerve tissue (53) was not detected in cell surface or in intracellular membranes (data not shown).

Insulin Redistributes Caveolin in the Muscle Fiber—Recent studies in 3T3-L1 adipocytes have reported that insulin increases the amount of caveolin found in plasma membrane (28). To determine whether insulin also affects the distribution of caveolin in skeletal muscle, the abundance of GLUT4 and caveolin in intracellular and cell surface membranes before and after insulin treatment was next studied (Fig. 4). The *in vivo* administration of insulin (30 min after 10 units of insulin/kg of body weight, intravenously) caused an increased abundance of

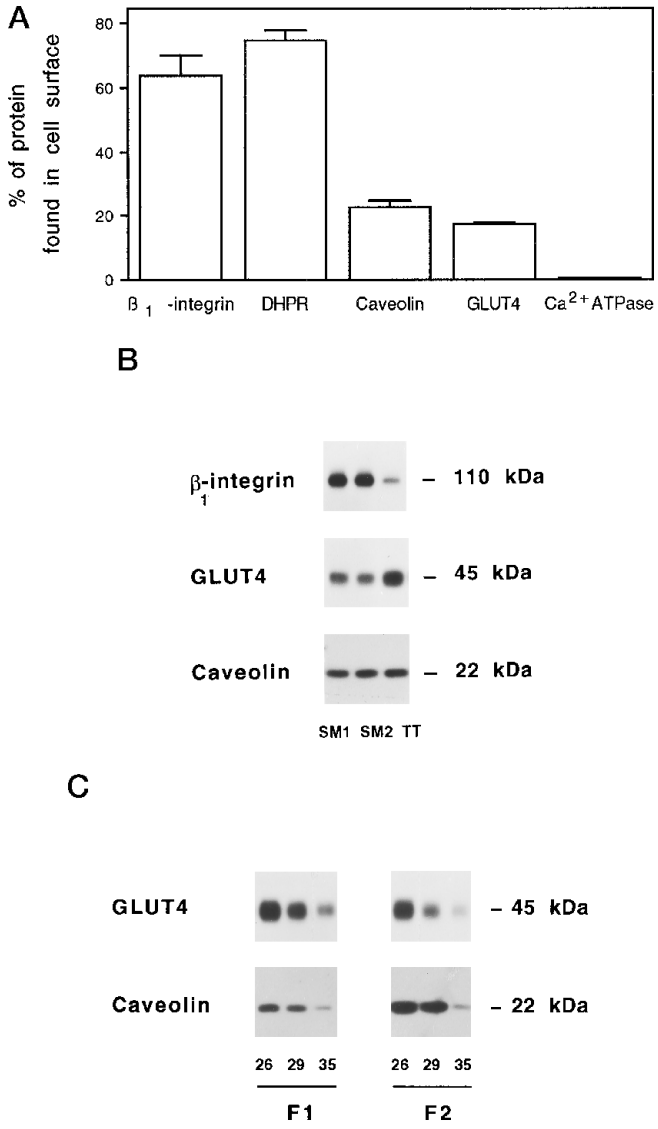

FIG. 3. **Caveolin and GLUT4 are mainly found in intracellular membranes in skeletal muscle.** The abundance of caveolin, GLUT4, β_1-integrin, dihydropyridine receptor and sarcoplasmic Ca^{2+}-ATPase in cell surface is shown as the percentage of each specific protein found in cell surface membranes (*A*) and the results are mean ± S.E. of four to six separate experiments. The abundance of caveolin, GLUT4, and β_1-integrin was assayed in surface membrane *fractions SM1*, *SM2*, and *TT* (*B*) and in intracellular membranes *26F1*, *29F1*, *35F1*, *26F2*, *29F2*, and *35F2* (*C*) from rat skeletal muscle. The distribution of the caveolin, GLUT4, β_1-integrin, dihydropyridine receptor, and sarcoplasmic Ca^{2+}-ATPase was determined by immunoblot analysis by using specific antibodies (see "Experimental Procedures"). Equal amounts of membrane proteins (1 μg for GLU4 and β_1-integrin and 4 μg for caveolin) from the different fractions were laid on gels. Representative autoradiograms from four to seven experiments are shown in *B* and *C*. The autoradiograms presented in *B* and *C* were exposed for different time periods.

GLUT4 in cell surface membranes (*fraction 23F2*, Fig. 4). This pattern of changes in the distribution of GLUT4 in surface membranes was specific to the glucose transporter, and no effect of insulin was detected in the distribution of the surface markers such as β_1-integrin or dihydropyridine receptors (data not shown). Concomitant to the changes observed in cell surface domains, insulin treatment caused a marked decrease in the content of GLUT4 in intracellular vesicles derived from fractions 26F1, 29F1, and 35F1 (Fig. 4). No effect of insulin was detected in the GLUT4 content of intracellular fractions 26F2, 29F2, or 35F2 (Fig. 4).

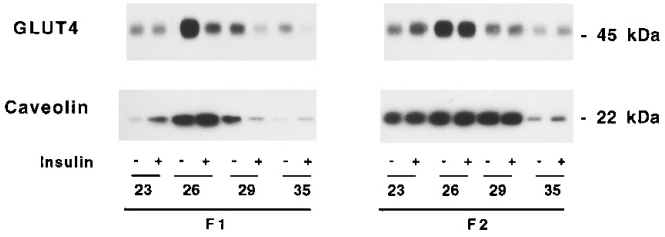

FIG. 4. **Insulin redistributes caveolin and GLUT4 in membrane fractions from skeletal muscle.** The abundance of caveolin and GLUT4 was assayed in cell surface membrane *fractions 23F1* and *23F2* and in intracellular membranes *26F1*, *29F1*, *35F1*, *26F2*, *29F2*, and *35F2* from control and insulin-stimulated muscles. The distribution of caveolin and GLUT4 was determined by immunoblot analysis by using specific antibodies. Equal amounts of membrane proteins (1 μg for GLUT4 and 4 μg for caveolin) from the different fractions were laid on gels. Representative autoradiograms, obtained after various times of exposure, from two to seven separate experiments are shown.

The abundance of caveolin was increased in response to insulin in fractions 23F1 (a cell surface membrane population) and 26F1 (an intracellular membrane fraction) (levels of caveolin in the insulin-treated group increased by 73 and 72% compared to control values in 23F1 and 26F1, respectively). Under these conditions, the levels of caveolin were markedly decreased in the insulin-treated group in intracellular fractions 29F1 and 35F1 (levels of caveolin decreased in response to insulin by 95 and 94% in 29F1 and 35F1, respectively) (Fig. 4). No alterations in the abundance of caveolin were substantiated in fractions 23F2 (a cell surface membrane fraction) and 26F2, 29F2 and 35F2 (from intracellular origin) (Fig. 4). These data suggest that insulin redistributes caveolin in a complex manner from some intracellular high density membrane fractions to intracellular lighter density membranes as well as to the cell surface.

Caveolin Does Not Colocalize with GLUT4 in Intracellular Membranes—Based on the similar pattern of distribution shown by caveolin and GLUT4 in cell surface and in intracellular membranes, we next studied whether these two proteins colocalized in membranes from skeletal muscle. In a first step, we isolated caveolin-rich membrane domains by a previously reported method (41). All of the caveolin was found in light-density fractions containing Triton-insoluble complexes (Fig. 5). Importantly, proteins characteristic of the plasma membrane such as the α_1 subunit of the Na^+-K^+-ATPase are excluded from this low density fraction (data not shown) indicating that this is a valid procedure for isolation of caveolin-rich membrane domains. Under these conditions, we detected a low level of overlapping between the sedimentation pattern of caveolin-rich membrane domains and GLUT4 (less than 10% of GLUT4 was found in caveolin-containing fractions) (Fig. 5).

We have identified two distinct pools of intracellular GLUT4-containing membranes in skeletal muscle based on their differing insulin response and whereas one pool (membrane fractions 26F2, 29F2, and 35F2) was insulin-insensitive, the other one (membrane fractions 26F1, 29F1, and 35F1) showed a marked decrease in GLUT4 content after insulin administration (Fig. 4). In order to determine whether intracellular caveolin and GLUT4 colocalize in the muscle fiber, vesicle immunoisolation assays were performed using antibody 1F8 (against GLUT4) coupled to acrylic beads, using insulin-sensitive (*i.e.* fraction 26F1) or insulin-insensitive (*i.e.* fraction 26F2) intracellular membrane fractions. Antibody 1F8 immunoadsorbed nearly 90% and 89% of total GLUT4 from the fractions 26F1 and 26F2, respectively (Fig. 6, *A* and *B*). Under these conditions, antibody 1F8 did not immunoadsorb caveolin (Fig. 6, *A* and *B*). We also analyzed whether some degree of colocalization

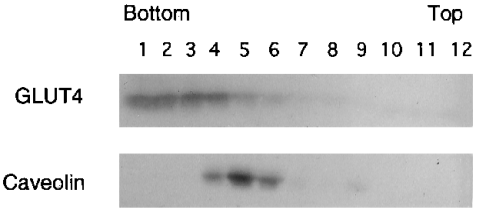

FIG. 5. **Subcellular localization of GLUT4 and caveolin in rat skeletal muscle.** Rat skeletal muscle was homogenized in buffer containing 1% Triton X-100, brought to 40% sucrose and overlaid with a linear 5–30% sucrose gradient lacking detergent. After centrifugation for 12–16 h at 39,000 rpm, 12 fractions were harvested. An equal volume of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting for the distribution of caveolin and GLUT4.

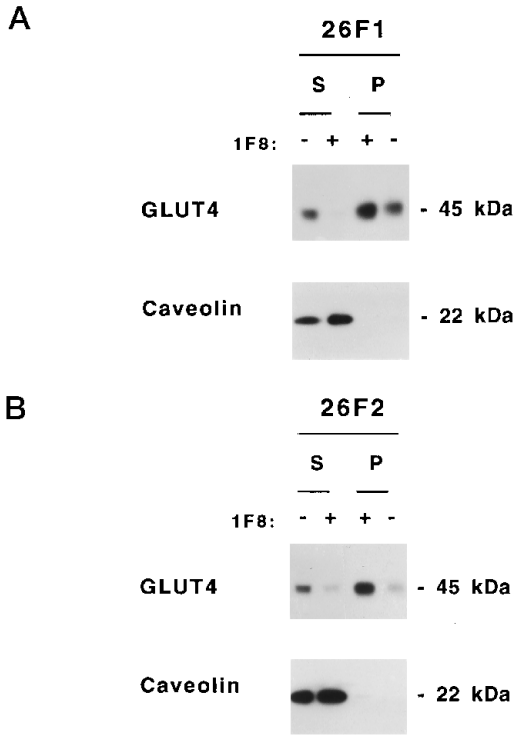

FIG. 6. **Absence of caveolin in intracellular insulin-sensitive and insulin-insensitive GLUT4 pools.** Membrane vesicles *26F1* (insulin-sensitive GLUT4 pool) (*A*) and membrane vesicles *26F2* (insulin-insensitive GLUT4 pool) (*B*) obtained from from nonstimulated skeletal muscle were incubated with (+) or without (−) antibody 1F8. After the incubation, the adsorbed (*P*) and nonadsorbed (*S*) fractions were electrophoresed and immunoblotted to determine the abundance of caveolin and GLUT4. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure, are shown.

between caveolin and GLUT4 was detected in insulin-sensitive GLUT4 pools after *in vivo* insulin treatment. To this end, immunoadsorption assays were performed by incubating fractions 26F1 and 29F1 (insulin-sensitive intracellular membrane fractions) from control and insulin-treated muscles with immobilized antibody 1F8. Antibody 1F8 immunoadsorbed smaller amounts of GLUT4 in the insulin-treated group than in control when starting with fraction 26F1 (data not shown). Under these conditions, caveolin was absent from these vesicle population (data not shown). Identical results were obtained with fraction 29F1 (data not shown).

In other studies, monoclonal antibody 3F8 directed against SCAMPs was also used in vesicle immunoisolation of insulin-sensitive and insulin-insensitive GLUT4 pools. Antibody 3F8 immunoadsorbed nearly 38 and 47% of total SCAMP 37 and 18 and 31% of total GLUT4 from fractions 26F1 and 26F2, respectively (data not shown). However, under these conditions, no caveolin was specifically immunoadsorbed (data not shown).

DISCUSSION

The results of this study demonstrate an abundant expression of caveolin in muscle and its regulation by myogenesis, aging and high fat feeding. We have also found that most of caveolin is distributed intracellularly in skeletal muscle under basal conditions and that acute insulin administration redistributes caveolin from intracellular high density membranes to the cell surface and to intracellular lighter density membranes. These results suggest that caveolin participates in vesicular traffic in skeletal muscle in an insulin-regulatable manner. However, intracellular caveolin does not colocalize with SCAMP proteins, markers of the general cell surface recycling system (54, 55), or with the GLUT4 glucose transporter isoform. In consequence, the vesicular traffic machinery in which caveolin participates is different than the system associated with SCAMPs and, at least in skeletal muscle, it is also unrelated to the vesicular traffic of GLUT4.

Our data emphasize the importance of understanding the biological role of caveolin in skeletal muscle. This is based on two findings, namely that caveolin is abundantly expressed in skeletal muscle and that its expression is subjected to regulation. That muscle is an abundant source of caveolin is substantiated by the fact that the expression of caveolin in this tissue, when expressed per gram of tissue, accounts for 10% of values detected in adipose tissue, which is the most abundant source of caveolin (28). We have also demonstrated that aging and high fat feeding, two situations characterized by muscle insulin resistance (56–58) caused an enhancement of caveolin levels in skeletal muscle. The biological impact of the up-regulation of caveolin with aging or high fat feeding is unknown and the possible implication of caveolin up-regulation and muscle insulin resistance remains undetermined.

We have also observed that the differentiation of L6E9 cells into myotubes is associated with decreased cellular levels of caveolin. This does not necessarily mean a decrease in abundance of caveolar organelles during differentiation. In this regard, it has very recently reported a novel homologue of caveolin termed M-caveolin, which is expressed exclusively in skeletal muscle and heart and its expression is induced upon muscle differentiation in C2C12 cells (59). The mechanisms by which caveolin is abundantly expressed in the adult rat skeletal muscle but not in cultured myotubes deserves further study.

Caveolin is an integral part of the striated coat of caveolae, *i.e.* non-clathrin-coated invaginations of the plasma membrane. Caveolin seems to internalize from caveolae to intracellular compartments in response to okadaic acid (60). Cholesterol oxidation also causes the movement of caveolin from caveolae to the Golgi apparatus (61) in the absence of changes in the number or morphology of caveolae. This together with the observation that caveolin is an integral component of trans-Golgi network-derived transport vesicles (62) supports the view that caveolin is a component of the molecular machinery of vesicular transport. In this regard, we have found that in skeletal muscle, caveolin is mainly distributed intracellularly. Under basal conditions, 77% of all caveolin found in muscle membranes was detected intracellularly. This distribution pattern is similar to the distribution of SCAMP proteins (63) and to GLUT4 glucose transporter in skeletal muscle. The distribution of caveolin in intracellular membranes from rat skeletal muscle suggests a functional role in vesicular transport.

Under our experimental conditions, we found that *in vivo* insulin administration caused a marked redistribution of caveolin in skeletal muscle. Thus, caveolin moved from high density intracellular membranes to the cell surface and to intracellular membranes of lower density. This is in keeping with previous observations in 3T3-L1 adipocytes indicating that insulin increased the amount of caveolin present in the plasma membrane and a decrease in the amount of caveolin associated with low density microsomes (28). This pattern of insulin-induced redistribution of caveolin is fairly similar to the effect of insulin on the distribution of SCAMPs in skeletal muscle (63). However, the redistribution of caveolin was markedly different than the insulin-induced translocation of GLUT4. The biological role of the insulin-triggered redistribution of caveolin in skeletal muscle deserves further study.

It is important to determine whether caveolin and GLUT4 interact in the muscle fiber. This aspect has been studied in this report in a number of ways. Caveolin-rich membrane domains were isolated from rat skeletal muscle which revealed that less than 10% of total GLUT4 overlapped with caveolin-containing fractions. Additionally, the colocalization of GLUT4 and caveolin in intracellular membranes was done by vesicle immunoadsorption analysis. In these studies we used two distinct intracellular GLUT4 pools previously obtained from rat skeletal muscle (63): one intracellular GLUT4 pool that is insulin-sensitive since insulin administration causes a large depletion of this pool, and a second pool that is insulin-insensitive in terms of GLUT4 content. Vesicle immunoisolation assays clearly indicated that caveolin was absent from these distinct intracellular GLUT4-containing membranes obtained from muscle under basal or insulin-stimulated conditions. This allows us to conclude that caveolin is not an intermediate in intracellular trafficking of GLUT4 in skeletal muscle. If the overlapping found between GLUT4 and caveolin-rich membrane domains was interpreted as real colocalization between GLUT4 and caveolin, these data together with results obtained from vesicle immunoisolation analysis would be consistent with the idea that caveolin-rich membrane domains act as transient intermediates in GLUT4 endocytosis and/or exocytosis in muscle. This hypothesis requires extensive experimental work.

In any case, our studies indicate that caveolin is not an intermediate in intracellular trafficking of GLUT4 in skeletal muscle, as found in isolated rat adipocytes (29) and in contrast to what has been suggested in 3T3-L1 adipocytes (28). The reason for this discrepancy is at present unknown but it should be mentioned that 3T3-L1 adipocytes also shows marked differences compared with isolated rat adipocytes in the trafficking of GLUT1 and GLUT4, and whereas GLUT1 and GLUT4 largely colocalize in 3T3-L1 they are completely seggregated in rat adipocytes (64–66).

We also searched for colocalization of caveolin with SCAMPs proteins. SCAMPs are expressed in rat skeletal muscle where they show an intracellular distribution (63). Insulin has been found to alter the cellular distribution of SCAMPs in adipocytes and in skeletal muscle and a variable extent of colocalization has been reported between intracellular SCAMPs and GLUT4 in adipocytes (18, 19, 63). To determine whether SCAMPs and caveolin colocalize in skeletal muscle membranes, intracellular SCAMP-containing vesicles were immunoisolated with immobilized antibody 3F8. Under conditions, in which we immunoisolated near 40–50% of SCAMPs, caveolin was absent from the immunoprecipitates. In consequence, we conclude that there is a seggregation of SCAMPs and caveolin in intracellular membranes from rat skeletal muscle. In summary, based on the pattern of cellular distribution caused by insulin and on the lack of colocalization between caveolin and GLUT4 or SCAMPs, we propose the activity of, at least, three separate insulin-regulated mechanisms for vesicular transport in skeletal muscle. One system is characterized by the presence of caveolin, the second is characterized by the presence of SCAMPs and the third is involved in the recycling of GLUT4 glucose carriers.

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